Biochem. J. (1985) 231, 53–57 (Printed in Great Britain)

Purification, from cultured human choriocarcinoma cells, of a 75000-M_r protein reacting with antibodies to a synthetic peptide based on a cloned human endogenous provirus nucleotide sequence

Ale NÄRVÄNEN

*Department of Virology, University of Helsinki, Haartmaninkatu 3, 00290 Helsinki, and Labsystems Research Laboratories, Pulttitie 8, 00810 Helsinki, Finland

We previously detected in cultured choriocarcinoma cells a 75000-M_r polypeptide defined by immunoblotting with antibody to a synthetic peptide Sp23 (Cys-Glu-Asn-Pro-Ser-Gln-Phe-Tyr-Glu-Asp-Leu) based on a cloned human endogenous proviral nucleotide sequence. On immunohistological staining, anti-Sp23 stains antigen(s) in the syncytiotrophoblasts of first-trimester placentas and in renal-cell adenocarcinoma tissues. The present report describes purification to homogeneity of the protein from cultured choriocarcinoma cells. The procedure involves extraction with non-ionic detergent and h.p.l.c. using, sequentially, gel-permeation, anion-exchange and reverse-phase columns. The yield was 110 μg/g of total choriocarcinoma-cell protein. The results indicate that the purified protein is a monomeric and relatively hydrophilic molecule of M_r 75000.

INTRODUCTION

Antibodies to synthetic peptides provide a powerful tool to identify novel proteins. Known gene sequences can be translated at the amino acid level, and selected sequences can be synthesized (Sutcliffe *et al.*, 1980). By using hybridization techniques, evidence has been presented that human DNA contains numerous retrovirus-related nucleotide sequences (Martin *et al.*, 1981; Bonner *et al.*, 1982; Callahan *et al.*, 1982; Noda *et al.*, 1982; Beneviste & Todaro *et al.*, 1974; Cohen & Murphey-Corb, 1983; May *et al.*, 1983; O'Brien *et al.*, 1983; Rabson *et al.*, 1983; Repaske *et al.*, 1983; O'Connell & Cohen, 1984; Steele *et al.*, 1984; Mager & Henthorn, 1985). One of the sequences, that of a defective endogenous provirus, was cloned from a human recombinant-DNA library by using as a probe an endogenous chimpanzee retroviral *pol* gene highly related to that of baboon endogenous retrovirus (BaEV) (Bonner *et al.*, 1982). This cloned sequence, termed HC-20 (or endogenous retrovirus-1; *erv*-1) contains *gag* and *pol* genes, which are significantly related to those of both Moloney murine leukaemia virus (Mo-MuLV) and BaEV. A peptide (Cys-Glu-Asn-Pro-Ser-Gln-Phe-Tyr-Glu-Arg-Leu) was inferred and synthesized from the nucleotide sequence of this provirus at locus *erv*-1. The peptide used to raise antibodies contained an additional *N*-terminal cysteine residue, added to facilitate coupling of the peptide to the carrier protein. This peptide (Sp-23), minus the extra cysteine residue, has partial sequence homology with MuLV and BaEV p30 proteins. Rabbit antibody against Sp-23 detects, on immunoblotting, a 75000-M_r protein both in first-trimester human placental syncytiotrophoblasts and in cultured choriocarcinoma cells. Immunoperoxidase staining of tissue sections with anti-Sp23 gave a strong reaction in the syncytiotrophoblastic cells of all early (< 15 weeks) placentas examined, but was negative for older placentas and for all other normal tissues studied (Suni *et al.*, 1984). In addition, anti-Sp23 detected a 75000-M_r polypeptide in human renal-cell-adenocarcinoma (hypernephroma) tumour tissues. Sections of tumour tissues, but not of normal kidneys or of other tumours, were found to be positive on immunoperoxidase staining (Wahlström *et al.*, 1985). The present report describes the purification of the anti-Sp23-reactive protein to homogeneity from cultured choriocarcinoma cells.

EXPERIMENTAL

Cultured choriocarcinoma cells

Human choriocarcinoma JEG-3 cells (A.T.C.C. HTB 36; American Type Culture Collection, Rockville, MD, U.S.A.), known to secrete human chorionic gonadotropin, were grown in roller bottles (1350 cm^2) as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 10% (v/v) foetal-bovine serum. Under these conditions, 1–2% of the cells had a syncytiotrophoblastic morphology; the rest of the cells were cytotrophoblast-like.

In the final purification procedure, JEG-3 cell layers were washed three times with phosphate-buffered saline (10 mM-phosphate/150 mM-NaCl, pH 7.4) and harvested with a rubber policeman by using 1% (w/v) Triton X-114 in TEN buffer [50 mM-Tris/HCl (pH 7.4)/10 mM-EDTA/150 mM-NaCl]. Insoluble material was removed by centrifugation at 10000 *g* for 15 min at +4 °C.

Triton X-114 two-phase separation

The procedure described by Bordier (1981) was followed. Briefly, Triton X-114 (Sigma, St. Louis, MO,

Abbreviations used: BaEV, baboon endogenous retrovirus; Mo-MuLV, Moloney murine leukaemia virus; MEM, (Eagle's) minimal essential medium; f.p.l.c., fast protein liquid chromatography; SDS, sodium dodecyl sulphate; TFA, trifluoroacetic acid.

* Address for correspondence and reprint requests.

U.S.A.) was precondensed and used at a 1% (v/v) concentration in TEN buffer. JEG-3 cells were grown as monolayers to confluency and washed as described above. The dishes were cooled on ice and 1 ml of ice-cold 1% Triton X-114 was added. The dishes were tilted gently for 10 min and the material was collected into a Minifuge tube. Insoluble material was sedimented and the supernatant was phase-separated as described by Bordier (1981). All three fractions, insoluble material (collected in 50 μl), detergent phase (50 μl) and water phase (950 μl), were analysed by immunoblotting with anti-Sp23 as antibody.

Gel filtration

Crude fractionation was carried out by using a TSK 3000 gel-filtration column (21.5 mm × 300 mm) (Toyo Soda, Tokyo, Japan) and h.p.l.c. (Varian 5000 liquid chromatograph, Varian UV-100 detector and Varian 4270 integrator). The running buffer was 50 mM-Tris/HCl, pH 6.5, in 150 mM-NaCl, the flow rate 3 ml/min and the run was monitored by recording A_{280}. Fractions (3 ml each) were collected and tested with the immunoblotting technique with anti-Sp23 as antibody.

Positive fractions were pooled, freeze-dried and dissolved in $\frac{1}{6}$ vol. of distilled water. The solvent was changed to 20 mM-Tris/HCl, pH 7.5, and a Pharmacia PD 10 gel-filtration column was used. A single 3 ml fraction was collected.

Anion-exchange chromatography

A Pharmacia Mono Q column and Pharmacia f.p.l.c. apparatus (GP-250 gradient programmer, P-500 pumps and UV-1 single-path monitor) were used in anion exchange chromatography, the eluate being monitored at 280 nm. The running buffer was 20 mM-Tris/HCl, pH 7.5 (buffer A). The column was first eluted with 10% (v/v) buffer B [20 mM-Tris/HCl (pH 7.5)/560 mM-NaCl], and then the proteins were eluted with a linear 10–100%-buffer-B gradient for 36 min at a flow rate of 1 ml/min. Fractions (1 ml) were collected and tested described as above.

Reverse-phase chromatography

The last step in the purification procedure was a run with the Pharmacia Pro-RPC reverse-phase C-18 column with the Varian h.p.l.c. system. The running buffer was 0.1% TFA in distilled water (buffer C) and the bound proteins were eluted with acetonitrile containing 0.08% TFA (buffer D). The program was: 0–7 min, 0% D; 7–10 min, 30% D; 10–20 min, 50% D; 20–23 min, 100% D; 23–27 min, 100% D; and 27–30 min, 0% D. The flow rate was 0.7 ml/min and the wavelength 218 nm. Protein-containing peaks were collected manually and tested as described above. The degree of purification was tested by SDS/polyacrylamide-gel electrophoresis.

Polyacrylamide-gel electrophoresis and immunoblotting

For SDS/polyacrylamide-gel electrophoresis, portions of the fractions were mixed with 1 vol. of Laemmli's (1970) sample buffer. The proteins were separated by SDS/polyacrylamide-gel electrophoresis, with 10%-(w/v)-polyacrylamide slab gels as described by Laemmli (1970), under reducing conditions. Coomassie Brilliant Blue (0.2% (v/v)) was used to stain proteins in the gels. The gels were destained with 10% (v/v) acetic acid.

For immunoblotting the proteins were transferred electrophoretically from the gel to nitrocellulose sheets and immunoblotted as described by Towbin *et al.*, (1979), modified as described by Vartio *et al.* (1982). Proteins were immunostained with rabbit anti-Sp23 antiserum (Suni *et al.*, 1984) at a 1:500 dilution in TEN-Tx buffer [50 mM-Tris/HCl (pH 7.0)/5 mM-EDTA/150 mM-NaCl/0.05% Triton X-100]. The immunoreactive polypeptide band was detected with peroxidase-conjugated anti-rabbit IgG (Dako, Copenhagen, Denmark).

RESULTS

In agreement with previous observations, immunoblotting with anti-Sp23 detected a single band at M_r 75000 from polypeptides separated by SDS/polyacrylamide-gel electrophoresis and transferred to nitrocellulose paper. This technique, as well as SDS/polyacrylamide-gel electrophoresis followed by protein staining, were used to monitor all purification steps.

In order to determine whether the protein was hydrophobic or hydrophilic, the Triton X-114 two-phase system was applied. As shown in Fig. 1, all of the 75000-M_r

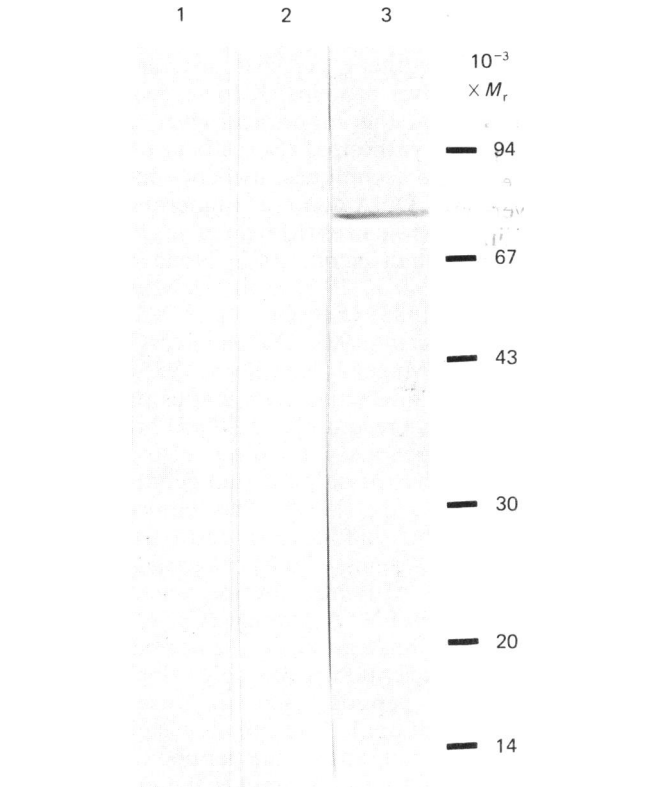

Fig. 1. Triton X-114 phase separation of cultured choriocarcinoma cells

The phase separation was carried out as described in the Experimental section, and the fractions were analysed by SDS/polyacrylamide-gel electrophoresis followed by immunoblotting with anti-Sp23 as antibody. Insoluble material (lane 1) and the lower phase (hydrophobic; lane 2), which are obtained in small volumes, were analysed as such. The upper phase (hydrophilic; lane 3) was concentrated 10-fold by freeze-drying. Anti-Sp23 reactivity can be seen only in lane 3. The M_r values for the marker proteins are indicated on the right.

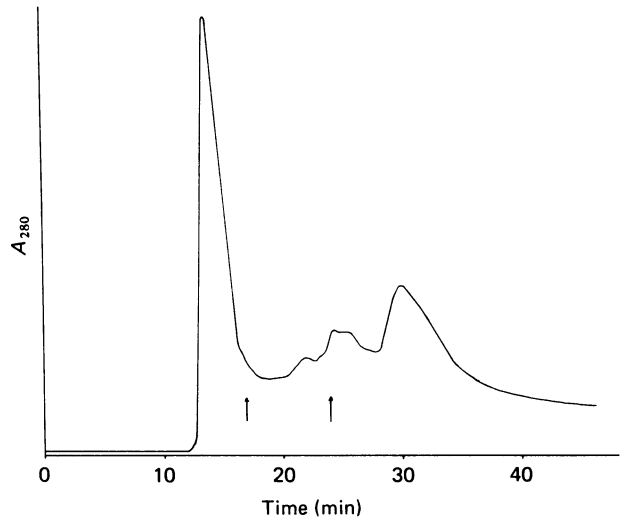

Fig. 2. Gel-filtration h.p.l.c. (TSK 3000) chromatogram of Triton X-114-extracted choriocarcinoma cells

The running buffer was 50 mm-Tris/HCl, pH 6.5, containing 150 mM-NaCl, and the flow rate was 3 ml/min. The run was monitored at 280 nm. The 75000-M_r peak, identified by using anti-Sp23 in immunoblotting, is found between 17 and 24 min, as shown by the arrows. The first large peak is mostly due to Triton X-114.

polypeptide separated into the hydrophilic phase. On the basis of this result, the JEG choriocarcinoma cells, used as the source for the purification, were treated as follows. The cell layers were washed three times with phosphate-buffered saline and then dissolved in ice-cold buffer containing 1% Triton X-114. Insoluble material was removed by low-speed centrifugation (10000 *g*, 20 min, +4 °C). No anti-Sp23 reactivity was found to be sedimented.

Molecular sieving was carried out by using an h.p.l.c. gel-permeation column. As seen in Fig. 2, the 75000-M_r reactivity migrated as a single broad peak but could be separated from small-sized proteins. The large peak in Fig. 2 is mostly due to the Triton X-114 detergent molecules, which absorb at 260 nm (Tiller *et al.*, 1984). The position of albumin (M_r 67000) in fractions 20–24 min indicates that the 75000-M_r component (fractions 17–24 min) is a monomeric protein. The positive fractions were pooled and processed for anion-exchange h.p.l.c. As seen in Fig. 3, the 75000-M_r protein (reactive with anti-Sp23 on immunoblotting) was eluted as a single peak at 25% buffer B (150 mM-NaCl). SDS/polyacrylamide-gel electrophoresis of the active peak and neighbouring fractions is shown in Fig. 4, and in Fig. 5 comparison is made with the degree of purification at the other purification steps. The major contaminant at this stage was a polypeptide of M_r 30000. The final purification was achieved by using reverse-phase h.p.l.c. (Fig. 6). The large peak containing the 75000-M_r protein was eluted at 45% buffer B (45% acetonitrile). SDS/polyacrylamide-gel electrophoresis of the purified protein (Fig. 4, track 3) indicates that the 75000-M_r protein had been purified to homogeneity. Immunoblotting with anti-Sp23 shows that it specifically reacts with the purified protein.

The yield of the purified protein, as estimated from the h.p.l.c. chromatograms and from SDS/polyacrylamide-gel electrophoresis of diluted samples, was found to be about 150 μg per 20 roller cultures (about 1.35 g total protein in the cell layer).

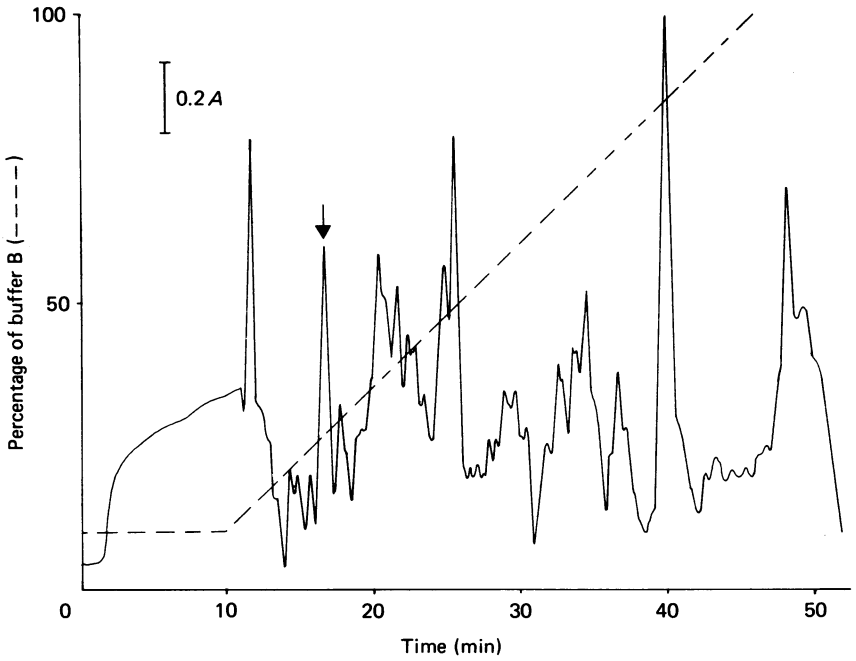

Fig. 3. Anion-exchange h.p.l.c. (Mono Q) chromatographic run of the pooled positive gel-filtration fractions

The running buffer was 20 mM-Tris/HCl, pH 7.5, and proteins were eluted with a 36-min linear gradient (----) from 10 to 100% buffer B (20 mM-Tris/HCl, pH 7.5, containing 560 mM-NaCl). The column was first eluted with 10% buffer B for 10 min. The flow rate was 1 ml/min and the run was monitored at 280 nm (——). The 75000-M_r protein, identified by using anti-Sp23 in immunoblotting, was eluted as a single peak at 25% buffer B (about 150 mM-NaCl), as shown by the arrow.

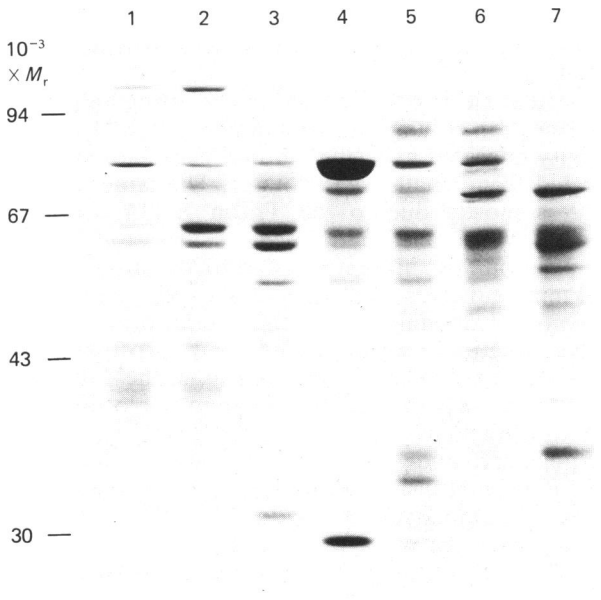

Fig. 4. SDS/polyacrylamide-gel electrophoresis of fractions collected from anion-exchange chromatography

Portions (25 μl) were taken from seven fractions corresponding to the peaks in Fig. 3 at 14.5, 14.9, 15.8, 17.0, 19.1, 20.8 and 22.1 min. The fraction containing the 75000-M_r protein is seen in track 4. M_r values are indicated on the left.

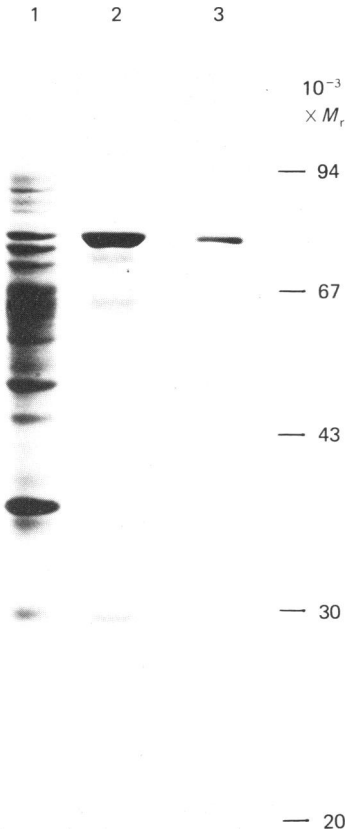

Fig. 5. SDS/polyacrylamide-gel electrophoresis of the 75000-M_r protein-containing peaks of the three sequential h.p.l.c. chromatography steps

Track 1, positive peak fraction, gel filtration (Fig. 2); track 2, positive peak fraction, anion exchange (Fig. 3); track 3, positive peak fraction, reverse-phase chromatography (Fig. 6). M_r values are indicated on the right.

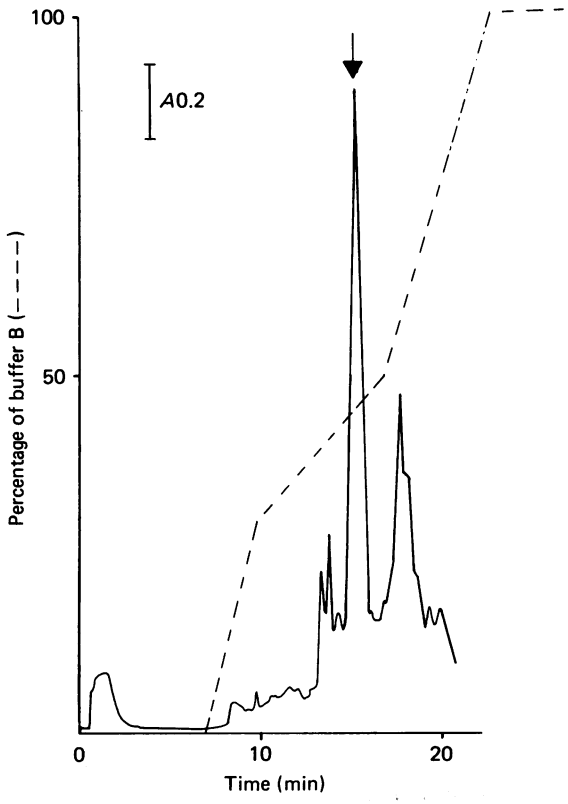

Fig. 6. Reverse-phase h.p.l.c. chromatogram of the positive fraction of the anion-exchange run

The running buffer (C) was 0.1% TFA in distilled water and the proteins were eluted with acetonitrile containing 0.08% TFA as buffer D, with the following gradient (- - - -): 0–7 min, 0% D;7–10 min, 30% D; 10–20 min, 50% D; 20–23 min, 100% D; 27–30 min, 0% D. The flow rate was 0.7 ml/min and the run was monitored at 218 nm (——). The M_r-75000 protein was eluted at 45% buffer D in a single peak, as shown by the arrow.

DISCUSSION

The present results indicate that the 75000-M_r component, defined by antibodies to the synthetic peptide Cys-Glu-Asn-Pro-Ser-Glu-Phe-Tyr-Glu-Asp-Leu, is a hydrophilic monomeric protein. The yield of the protein represents 0.02% of the total cellular protein in cultured JEG-3 choriocarcinoma cells. This value is a minimum estimate. At the present it is not possible to determine the absolute concentration of the 75000-M_r protein in these cells.

Monitoring of the purification with the immunoblotting method suggested that on anion-exchange h.p.l.c. and reverse-phase h.p.l.c. the recovery was about 90%, less in gel-permeation h.p.l.c., and additional losses apparently occurred at least at the buffer-exchange step. These

results reinforce the view that h.p.l.c. provides a powerful tool for purification of relatively hydrophilic and high-M_r proteins. The results also indicate that the antigenity of this protein, at least with antibodies raised against synthetic peptides, is also retained in the reverse-phase h.p.l.c. procedure in which relatively severe conditions are used (in the present case, 45% acetonitrile at pH 2).

Purification of the 75000-M_r protein was carried out for several reasons. Firstly, it was to produce material for sequencing to verify its retroviral nature and to use the sequence information at the nucleotide level for cloning of the 75000-M_r-protein gene(s). It should be noted that although the synthetic peptide was deduced from a cloned human retroviral sequence HC-20, this may be a 'silent' gene, as it lacks the 5′ LTR promoter sequence (Bonner *et al.*, 1982). This is supported by our preliminary findings with cloned HC-20 sequence as a probe, which has failed in Northern-blotting experiments to detect mRNA in choriocarcinoma cells. Thus the antibodies to the synthetic peptide probably detect a product of a related active gene. Some preliminary experiments have indicated that the *N*-terminus of this protein is blocked. Secondly, the purified protein may be used to raise mono- and poly-clonal antibodies to be used in the immunochemical characterization of the 75000-M_r protein and to set up quantitative immunoassays for it. Our previous findings on the exclusive expression of anti-Sp23 reactivity (75000-M_r polypeptide in immunoblotting) and specific immunohistochemical staining in renal-cell-adenocarcinoma tissues (T. Wahlström, A. Närvänen, J. Suni, R. Pakkanen, T. Lehtonen, E. Saksela, A. Vaheri, T. Copeland, M. Cohen & S. Oroszlan, unpublished work) suggests that immunoassay of the M_r-75000 protein may provide a useful tumour marker.

I thank Professor Antti Vaheri for continuous support and advice, Dr. Raimo Pakkanen for helpful comments, Dr. Stephen Oroszlan for anti-Sp23 antibody, and Mrs. Leena Kostamovaara and Mrs. Anja Virtanen for technical assistance. This work was supported in part by the Medical Research Council of the Academy of Finland.

Received 18 March 1985/14 May 1985; accepted 5 June 1985

REFERENCES

Benveniste, R. E. & Todaro, G. J. (1974) Proc. Natl. Acad. Sci. U.S.A. **71**, 4513–4518
Bonner, T. I., O'Connell, C. & Cohen, M. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 4709–4713
Bordier, C. (1981) J. Biol. Chem. **256**, 1604–1607
Callahan, R., Drohan, W., Tronick, S. & Schlom, J. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 5503–5507
Cohen, J. C. & Murphey-Corb, M. (1983) Nature (London) **301**, 129–132
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Mager, D. L. & Henthorn, P. S. (1985) Proc. Natl. Acad. Sci. U.S.A. **81**, 7510–7514
Martin, M. A., Bryan, T., Rasheed, S. & Khan, A. S. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 4892–4896
May, F. E. B., Westley, B. R., Rochefort, H., Buetti, E. & Diggelman, H. (1983) Nucleic Acids Res. **11**, 4127–4139
Noda, M., Kurihara, M. & Takano, T. (1982) Nucleic Acids Res. **10**, 2865–2878
O'Brien, S. J., Bonner, T. I., Cohen, M., O'Connell, C. & Nash, W. G. (1983) Nature (London) **303**, 74–77
O'Connell, C. & Cohen, M. (1984) Science **226**, 1204–1206
Rabson, A. B., Steele, P. E., Garon, C. F. & Martin, M. A. (1983) Nature (London) **306**, 604–607
Repaske, R., O'Neill, R. R., Steele, P. E. & Martin, M. A. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 678–682
Steele, P. E., Rabson, A. B., Bryan, T. & Martin, M. A. (1984) Science **225**, 943–947
Suni, J., Närvänen, A., Wahlström, T., Aho, M., Pakkanen, R., Vaheri, A., Copeland, T., Cohen, M. & Oroszlan, S. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 6197–6201
Sutcliffe, J. G., Shinnick, T. M., Green, N., Liu, F.-T., Niman, H. L. & Lerner, R. A. (1980) Nature (London) **287**, 801–805
Tiller, G. E., Mueller, T. J., Dockter, M. E. & Struve, W. G. (1984) Anal. Biochem. **141**, 262–266
Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
Vartio, T., Zardi, L., Balza, E., Towbin, H. & Vaheri, A. (1982) J. Immunol. Methods **55**, 309–318
Wahlström, T., Närvänen, A. M., Suni, J., Pakkanen, R., Lehtonen, T., Saksela, E., Vaheri, A., Copeland, T., Cohen, M., and Oroszlan, S. (1985) Int. J. Cancer, in the press